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(57) Abstract

Dietetically improved bakery products having a content of viable microorganisms which is in the range of 10^3 to 2 x 10^{10} per g and a method of preparing such bakery products. The improved bakery products include ryebread, wheat flour-containing bread products or cakes, muffins and scones.

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BAKED PRODUCT CONTAINING VIABLE MICROORGANISMS AND PROCESS FOR PREPARING SAME

FIELD OF INVENTION

The present invention provides baked products containing dietetically desirable viable microorganisms and a process for preparing such products.

TECHNICAL BACKGROUND

Under normal conditions, the gastrointestinal tract of animals including humans is colonized by a numerous and diverse population of indigenous microorganisms. This naturally occurring intestinal microflora plays a significant role in maintaining the hosting macroorganism in a healthy condition due to several ecological effects of this microflora. First of all an intact intestinal flora will constitute a barrier against colonization of pathogenic organisms passing the gastrointestinal tract and thereby provide a natural protection against microbial diseases. Secondly, the indigenous microflora produce a number of enzymes and other metabolites which may supplement the digestion of nutrients which is caused by the natural digestive enzymes of the macroorganism.

Under certain conditions, however, the number of these naturally occurring intestinal microorganisms may be decreased or the beneficial balance between the species hereof may become less favourable to the host organism. Examples of such conditions are stress conditions, treatments with antimicrobial medicaments or inappropriate diets. These conditions may result in an increased susceptibility to acquiring diseases or in functional disturbances in the digestive tracts such as e.g. diarrhoea or constipation.

Under such less favourable conditions it may be advantageous to correct the disturbances of the natural microbial flora by administering to an individual suffering from such disturbances a large number of beneficial microorganisms which are capable of surviving or even colonizing the gastrointestinal tract. Examples of microorganisms which are currently administered with this purpose are lactic acid bacteria and yeast cultures.

The administration of beneficial microorganisms is not only considered advantageous for individuals having recognizable disturbances of their gastrointestinal flora. It is thus contemplated that even individuals without such recognizable disturbances may benefit from the administration as the above-defined function of their indigenous flora may be enhanced hereby.

Administration of dietetically effective microbial cultures may be via the diet such as by consumption of fermented dairy products or other food products including meat products and fermented vegetable products, containing viable lactic acid bacteria. However, the cultures may also conveniently be administered in the form of concentrates of the microorganisms, e.g. in the form of suitably formulated preparations including powders, granulates, tablets or capsules containing a high number of one or more species of the beneficial microorganisms. In general, such concentrated dosage forms are relatively expensive and hence, their distribution is still limited.

From a dietetic point of view it is advantageous to administer the beneficial microorganisms as a part of the normal diet. However, many humans dislike a constant intake of the above-mentioned fermented food products. Therefore, it is desirable to incorporate the beneficial microorganisms in types of food products which are consumed universally and regularly in considerable quantities by a majority of con-

sumers. In this respect, bread and other bakery products comply with this requirement.

However, bakery products are usually subjected to baking temperatures which will kill microorganisms except extremely thermotolerant species or thermotolerant forms of microorganisms. The present invention provides for the first time baked products which contain a dietetically desirable number of beneficial microorganisms as they have been defined above.

SUMMARY OF THE INVENTION

Accordingly, in one aspect the present invention relates to a method of preparing a bakery product containing viable microorganisms, comprising the steps of (1) preparing a baked product and cooling it to a temperature in the range of 0 to 70°C, (2) preparing a suspension of microorganisms, containing 10^7 to 10^{12} viable organisms per mL, (3) injecting into the baked product a volume of the suspension of microorganisms which is in the range of 2 to 20 mL per kg of product, to obtain the baked product having a content of viable microorganisms which is in the range of 10^3 to 2×10^{10} per g including contents which are in the range of 2×10^5 to 2×10^{10} per g.

In an interesting embodiment of the invention there is also provided a method wherein the suspension of microorganisms is injected by means of an apparatus for injecting a suspension of microorganisms into a baked product, comprising

(1) means for introducing the suspension into the baked product,

(2) means for containing the suspension to be injected,

(3) means for connecting the reservoir with said means for introducing the suspension,

(4) means for pneumatically transporting the suspension from the containing means and to the introducing means, and for pneumatically introducing the means for introducing the suspension into the baked product,

(5) means for adjusting the volume to be injected,

(6) means for maintaining the baked product to be injected in a position which allows the introduction of the suspension thereinto.

In another aspect, the present invention pertains to a baked product having a content of viable microorganisms which is in the range of 10^3 to 2×10^{10} per g.

DETAILED DISCLOSURE OF THE INVENTION

Doughs or batters for several types of bakery products contain viable microorganisms in large numbers. Thus, baker's yeast is added during the preparation of the dough for many types of wheat flour based bread types including rolls and buns. In these types of bakery products baker's yeast is added as a leavening agent and the leavening is a result of the production of gas from the metabolically active yeast cells.

In the preparation of certain bread products including rye-bread, viable bacterial cultures such as cultures of lactic acid bacteria are also added to the dough. The purpose hereof is to confer to the bread a desired flavour of organic acids and other aromatic compounds.

Traditionally, bread products in which wheat flour is partially or completely replaced by rye flour, are prepared from doughs comprising as a substantial ingredient a so-called "leaven" which is a culture of one or several species of

lactic acid bacteria grown in an aqueous suspension of flour. The lactic acid bacterial culture in such leavens may originate from the naturally occurring flora of lactic acid bacteria of the flour, or suitable commercial lactic acid bacterial starter cultures may be added. Suitable lactic acid bacterial species for use in doughs include homofermentative species which predominantly produce lactic acid when sugars are fermented by them, and heterofermentative species which when fermenting sugars, in addition to lactic acid produce other acids including acetic acid and possibly propionic acid.

When rye bread leavens are based on the propagation of indigenous lactic acid bacteria of the flour ingredient, a continuous process is normally used whereby a substantial amount of an outgrown leaven culture is used as an ingredient of a new leaven mixture.

However, in all types of bakery products where microorganisms are added during the preparation of the dough or batter, these organisms are inevitably killed during the baking step as a result of heat inactivation. Accordingly, fresh baked product do not normally contain viable microorganisms.

As it has been mentioned above, the present invention provides a method of preparing a bakery product containing a high number of dietetically desirable viable microorganisms which are introduced into the baked product by injecting into the baked product a suspension of the microorganisms.

As it is essential that the bakery product environment into which the live microorganisms are introduced allows the organisms to survive, the products should be cooled to a temperature which is not detrimental to the viability of the used microorganisms, prior to the introduction hereof. The thermotolerance, i.e. the highest temperature at which substantially none of the cells of a suspension of microorganisms as used herein are injured to an extent where they are

no longer capable of growing under suitable growth conditions, may vary considerably.

In general, a non-injuring temperature as defined above is in the range of 0 to 70°C. Accordingly, the baked product is cooled to a temperature within this range, the required cooling depending on the microorganism(s) used. Typically, the product is cooled to a temperature in the range of 20 to 65°C such as in the range of 50 to 60°C. In this context, it is important to note that a temperature gradient will occur during the cooling of a baked product. Depending on the desired site of injecting the microbial suspension the cooling is continued until a suitable temperature as defined above has been reached at that particular site.

In accordance with the present invention, a suitable microorganism is one which confers to the baked product a desired dietetically beneficial effect as it has been explained above and which microorganism is capable of maintaining its viability after injection into the product to a degree which results in the presence of a dietetically effective amount of viable organisms herein at the time of consumption of the baked product. Such a useful microorganism may be selected from a fungal species, a yeast species and a bacterial species.

One obvious prerequisite for obtaining the dietetically beneficial effect is to select microorganisms which when present in a baked product is capable of resisting the acidic conditions in the anterior parts of the gastrointestinal tract, in particular in the stomach and the anterior parts of the small intestines. Accordingly, particularly interesting microorganisms are microorganisms which inherently are tolerant to acidic conditions. However, it is also possible to use less acid tolerant organisms provided they are provided in a form where they are protected against gastrointestinal acidic conditions. Thus, the microorganism(s) may be coated or encapsulated by a suitable compound or suitable compounds

which are insoluble under acidic conditions such as in the stomach or the anterior parts of the small intestines but which is/are dissolved when present in an environment with a pH in the range of e.g. 5 to 10. Any suitable coating or encapsulation method known in the art may be used.

In accordance with the invention, the suspension of microorganisms being injected may comprise organisms belonging to the same species or it may comprise microorganisms which are a mixture of species of microorganisms. In advantageous embodiments of the invention, the microorganisms are organisms belonging to one or more species of lactic acid bacteria. This group of microorganisms having as a common characteristic the ability of producing lactic acid bacteria under microaerophilic or anaerobic conditions, comprise several genera including gram-positive organisms such as *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* and *Bifidobacterium*. The genus *Lactobacillus* include as typical examples the following species *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus delbrückii*, and *Lactobacillus hilgardii*. A typical *Bifidobacterium* species is *Bifidobacterium bifidum*. Among *Streptococcus* spp. *Streptococcus thermophilus* and *Streptococcus faecium* may be used in accordance with the present invention.

In addition to bacteria belonging to the above group of lactic acid bacteria certain *Propionibacterium* spp which are gram-positive anaerobic bacteria capable of fermenting lactate to propionate, including *Propionibacterium shermanii* may be used, optionally in combination with one or more lactic acid bacterial species such as e.g. *Lactobacillus acidophilus*, *Bifidobacterium bifidum* or *Streptococcus faecium*.

Several lactic acid bacterial species are commonly used as starter cultures in the manufacturing of fermented food products, such as dairy products, meat products, vegetable products and as mentioned above, in the manufacturing of

bread products. Frequently, such lactic acid bacterial starter cultures comprise a mixture of species. One typical example hereof is the use of a mixture of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in certain fermented milk products.

From a dietetic point of view, the use of a mixed culture of these species may be advantageous as *Lactobacillus acidophilus* is considered to be particularly adapted to remain viable or even colonize in the anterior parts of the gastrointestinal tract whereas *Bifidobacterium bifidum* which is a strictly anaerobic organism assumingly will colonize the posterior parts of the intestines where the E_h is low. It is contemplated that a dietetically beneficial effect may be obtained throughout the major part of the gastrointestinal tract by providing in a food product including the baked product according to the present invention, a mixture of lactic acid bacterial species each of which is particularly adapted to colonize in different parts of the gastrointestinal tract.

Accordingly, in one interesting embodiment of the present invention a useful suspension of microorganisms comprises a mixture of a microaerophilic lactic acid bacterium such as a *Lactobacillus* species and a strictly anaerobic lactic acid bacterial species including a *Bifidobacterium* species. However, under certain conditions it may be desirable to use only one species.

As it has been mentioned above, useful microorganisms may in the present context include yeast organisms. Typical examples hereof are *Saccharomyces cerevisiae* e.g. in the form of baker's yeast, and *Klyveromyces lactis*. In accordance with the invention, the culture of microorganisms to be injected into the bakery products may comprise a mixture of bacterial and yeast organisms.

In accordance with the invention, the microorganisms may be wild-type strains as isolated from their natural environment. It may, however, be advantageous to use microbial strains which have been improved be selection, by mutation or by genetic recombination.

From a hygienic point of view it is important that the injected suspension of microorganisms is biologically pure, i.e. it should only contain the desired microorganisms and no or only few foreign microorganisms as contaminating organisms. In bakery products contamination with undesired fungi are particularly serious as these organisms may be capable of multiplying under the conditions prevailing in bakery products including the low a_w. Accordingly, the term "suspension of microorganisms" as used herein denotes a substantially pure suspension of the desired microorganisms.

The suspension of microorganisms being injected suitably contains 10^7 to 10^{12} viable organisms per mL. In certain preferred embodiments, the content of microorganisms is in the range of 10^9 to 10^{11} per mL. Conveniently, the suspension of microorganisms is prepared from a concentrate of organisms containing 10^8 to 10^{13} organisms per g. Such concentrates may be in the form of a slurry or paste of freshly grown microbial cells. However, in industrial production it may be more convenient to prepare the suspension from a frozen or freeze-dried concentrate of the microorganism(s) optionally containing one or more cryoprotective substances.

The suspension of microorganisms may e.g. be prepared from a concentrate of organisms as defined above, by suspending 1 part of the concentrate of microorganisms in 5 to 20 parts of an aqueous medium comprising at least one microbial nutrient and at least one salt. In the present context, an aqueous medium may be one selected from distilled water, deionized water or tap water. It is well-known that in a concentrate of microorganisms as in the one presently defined, a certain proportion of the organisms may be in state of "stress" or

sublethal injury, where maintenance of their viability is dependent on the presence in their environment of certain nutrients.

Accordingly, the use of such sublethally injured microorganisms in the present invention may require the addition to the aqueous suspending medium of one or more nutrients which cannot be synthesized or utilized by them. In this context, suitable nutrients may be any nutrient normally used in culturing media for particular microorganisms or the suspending medium may be a commercial liquid culture medium such as e.g. the conventionally used tryptic soy broth medium, containing the required nutrients. Typically, such nutrients may be selected from yeast extract which i.a. contain a variety of vitamins, peptides and amino acids; a carbon source which e.g. may be selected from a monosaccharide, a disaccharide or a polysaccharide; and a vitamin or a mixture of vitamins. Furthermore, the suspension medium may suitably contain a salt or a mixture of salts. The salt(s) may be selected from an alkali metal salt such as NaCl or a phosphate and an alkaline earth metal salts including phosphates, chlorides or carbonates.

The suspension medium is preferably a sterile medium which may be provided by using only sterile ingredients or by subjecting the prepared medium to a treatment whereby contaminating microorganisms are killed or removed. Such treatments include heating at a temperature and for a period of time which results in a sterile or substantially sterile medium, and a filtering under conditions where microorganisms are separated from the medium.

Subsequent to the preparation of the suspension of microorganisms, the suspension may be left to stand at a temperature in the range of 0 to 40°C such as at ambient temperature for up till 6 hours such as about 15 to 120 minutes.

The volume of the suspension of microorganisms typically being injected into the baked product is in the range of 1 to 50 mL, preferably in the range of 2 to 20 mL, per kg of product. The appropriate volume depends on the type of bakery product. It is essential that the volume injected does not exceed the volume which can be absorbed by the product without conferring to the product undesirable wet and "sticky" spots which can be recognized by the consumer. It has thus been found that the introduction into ryebread loaf products of 3 to 15 mL per kg of product is suitable, provided this volume is distributed by multiple injections such as by means of a multiplicity of needles as it will be explained below. In particular, a volume which is in the range of 5 to 12 mL per kg such as 10 mL has proven to result in ryebread where the injected suspension is appropriately absorbed into the bread crumb.

The above volume ranges may be suitable for pieces of bakery products which have a weight being in the range of 0.2 to 2 kg. When the bakery products to be injected are in smaller pieces, such as buns or rolls having a weight e.g. in the range of 0.02 to 0.2 kg the volumes may conveniently be in the range of 0.1 to 2 mL, such as 0.25 to 1 mL.

In accordance with the invention, any bakery product having a crumb structure and consistency allowing the injection and absorption of the suspension of microorganism(s) may be subjected to the injection of such a suspension. Interesting bakery products include ryebread products, yeast-leavened bakery products and chemically leavened bakery products. Yeast-leavened bakery products include wheat flour based bread products in the form of wheat flour-containing loaves, buns or rolls, and pastry products. Among chemically leavened bakery products typical examples are cakes such as sponge cakes, pound cakes, scones and muffins.

The bakery products containing the viable microorganisms may be packaged for display in retail shops e.g. in air-tight polymer foil materials, optionally as evacuated packages. When the bakery product is one which is consumed in slices, the packaged product may be provided as slices.

In one preferred embodiment of the present invention, the suspension of microorganisms is injected into the baked and cooled product through a multiplicity of needles having a length which is in the range of 5 to 100 cm and a diameter which is typically in the range of 0.5 to 3.0 mm such as about 2 mm, said needles being connected to one or more reservoirs containing the microbial suspension(s). A suitable length of the needles depend on the dimension of the product to be injected. In useful embodiments the needle length is 10 to 40 cm.

The injection of the suspension of microorganisms into the bakery product may take place during introduction of the needles or it occur during the retrograde movements of the needles. Most conveniently, however, the injection may take place during introduction as this allows the inner space of the needles to become refilled with the suspension during the retrograde movement. The direction of the needles in relation to the bakery product to be injected may be varied e.g. depending on the outer dimensions of the product. As an example, a loaf of ryebread or a loaf of wheat flour bread may conveniently be injected longitudinally.

Although the injection of the microbial suspension may be carried out manually such as by a syringe or a multiplicity of syringes provided with a needle, the injection is preferably carried out by means of an apparatus comprising (1) means for introducing the suspension into the baked product, (2) means for containing the suspension to be injected, (3) means for connecting the reservoir with said means for introducing the suspension, (4) means for pneumatically transporting the suspension from the containing means

and to the introducing means, and for pneumatically introducing the means for introducing the suspension into the baked product, (5) means for adjusting the volume to be injected, and (6) means for maintaining the baked product to be injected in a position which allows the introduction of the suspension thereinto.

Conveniently, the means for introducing the suspension of microorganisms into the baked product comprise a multiplicity of needles having the above-defined dimensions. A suitable number of needles depends on the dimension of the baked product to be injected, a suitable number of needles typically being in the range of 2 to 20 such as e.g. in the range of 3 to 10. In certain useful embodiments, the needles are placed equidistantly.

Suitable means for containing the suspension(s) of microorganisms include metal, glass and plastic containers such as tanks, bottles or jars. When it is preferred to inject two or more different suspensions separately, the means may comprise a multiplicity of containers connected to different needles thus allowing simultaneous injection of separate microbial cultures. In advantageous embodiments, the means for pneumatically transporting the suspension of microorganism(s) is activated during the introduction into the baked product of the means for introducing the suspension.

As it is mentioned above, the suspension of microorganisms may advantageously be injected into the baked product during introduction of the needles. Accordingly, the apparatus may be constructed so that the means for introducing the suspension is filled or refilled with suspension during retrograde movements of the means.

In accordance with the invention, the means for adjusting the total volume to be injected into one piece of bakery product may be constructed so as to allow adjustment of a volume which is in the range of 2 to 20 mL, preferably in the range

of 3 to 15 mL such as in the range of 5 to 12 mL, e.g. a volume of about 10 mL. These volume ranges may be suitable for pieces of bakery products which have a weight being in the range of 0.2 to 2 kg. When the bakery products to be injected are in smaller pieces, such as buns or rolls having a weight e.g. in the range of 0.02 to 0.2 kg the volumes may conveniently be in the range of 0.1 to 2 mL, such as 0.25 to 1 mL.

As mentioned above, the present invention provides in a further aspect a baked product prepared by the method as described herein which product has a content of viable dietetically beneficial microorganisms as defined above, being in the range of 10^3 to 2×10^{10} per g. In preferred embodiments the content of microorganisms may suitably be in the range of 2×10^5 to 2×10^{10} per g including contents in the range of 2×10^4 to 2×10^9 per g.

The desired content of viable microorganisms depends on the selected type(s) of microorganisms but it should preferably be at a level where a dietetically effective amount of microorganisms is provided in a typical serving of the particular bakery product. As one example, about 100 g of bread may typically be served for a meal. Assuming that a dietetically beneficial amount of injected viable microorganisms per meal is about 1×10^8, the bread served should contain at least 10^6 viable microorganisms per g. Accordingly, the injected amount of microorganisms must be adjusted to result in such a desired minimum amount of viable microorganisms at any point of time between manufacturing and consumption.

Most microorganisms which may be used in accordance with the invention will not multiply during the above-defined period of time but their number may gradually be reduced. Accordingly, the initial amount of viable microorganisms, i.e. the amount hereof present in the baked product immediately following injection must be selected at a higher level than the desired beneficial amount at the time of consumption so as to

compensate for a possible reduction of viability during storage. Typically, the desired initial amount of viable microorganisms may be 5 to 100 times higher than the amount required to provide the dietetically effective amount at the latest possible time of consumption. Thus, as an example, a loaf of ryebread may therefore have an initial content of viable microorganisms which in the range of 5×10^8 to 10^9 per g.

As it has been mentioned above, the number of viable microorganisms in the subject baked product is defined as the number hereof capable of growing under optimal conditions of growth. The appropriate method of enumeration will depend on the particular type of microorganism(s). Typically, such methods may be conventional plate count methods using a growth medium suitable for the microorganisms used and incubating the medium inoculated with suitably diluted samples of the product under suitable atmospheric and temperature conditions and counting the number of colony forming units.

The invention is further illustrated in the following examples:

EXAMPLE 1

Ryebread with Lactobacillus acidophilus and Bifidobacterium bifidum

1. Preparation of "leaven mixture" ingredient for ryebread

Initially, a "mother leaven" is prepared by inoculating 10 g of a commercial culture of *Lactobacillus acidophilus* suspended in 200 mL of demineralized water into a mixture of 2 kg of coarse rye flour and 2 L of water and incubating the resulting mixture at 38°C for about 8 hours followed by the addition to the mixture of additional rye flour and water and continued incubation at 38°C. This is repeated on the 2nd day

and on the 3rd day after which the leaven is ready for use in bread manufacturing.

2. Manufacturing of ryebread loaves

A dough was prepared according to the following recipe:

Linseed	4.0 kg
Water for soaking the linseed	8.0 kg
Soybean shells	4.0 kg
Coarse rye flour	28.0 kg
Liquid leaven mixture	20.0 kg
Syrup	0.6 kg
Salt	0.6 kg
Breadcrumbs	1.6 kg
Risofarin™ 1)	0.6 kg
Water	22.0 kg
Baker's yeast	0.6 kg

1) A mixture of modified maize, rice and wheat starches

The ingredients except the soybean shells were mixed into a dough by kneading in a kneading apparatus for about 15 minutes and left to stand for about 15 minutes at a temperature of about 28 to 30°C.

In a subsequent step, the dough was portioned into 1100 g units being formed into loaf shape and covered with soybean shells and transferred to baking tins. The loaves were next left to rise for about 50 minutes before being transferred to an oven having an initial temperature of 270°C and into which steam was lead during the baking process. The baking time was about 75 minutes during which the temperature was gradually lowered to about 170°C.

After the baking step, the loaves were taken to a cooling tower in which air at a temperature of 5 to 7°C was circulated at a rate of 10.000 to 16.000 m^3 per hour. After cool-

ing for about 45 minutes, the temperature in the bread crumb was about 66°C.

3. Injecting a suspension of a mixture of Lactobacillus acidophilus and Bifidobacterium acidophilus

A suspension of microorganisms was prepared by suspending 1 g of a frozen culture of *Lactobacillus acidophilus* (Nu-trish™, Chr. Hansen's Laboratorium Danmark A/S, Hørsholm, Denmark) containing about $5x10^{11}$ viable organisms, 1 g of a frozen culture of *Bifidobacterium bifidum* (Nu-trish™, Chr. Hansen's Laboratorium Danmark A/S, Hørsholm, Denmark) containing about $8.3x10^{11}$ viable organisms in 10 g of suspension medium comprising 1 wt% of sterile yeast extract, 0.9 wt% of sterile NaCl and 98.1 wt% of sterile distilled water.

The resulting suspension was left to stand for up till 6 hours at a temperature of about 37°C prior to injecting about 10 mL per kg into the baked and cooled loaves by means of a multiplicity of needles having a length of about 40 cm and diameter of about 3 mm.

4. Number of surviving bacteria during storage of ryebread loaves at 20-25°C

The number of viable *Lactobacillus acidophilus* and *Bifidobacterium bifidum* was followed during storage at intervals of 2 days. Slices of the bread was homogenized in an aqueous medium and serial dilutions hereof was plated onto MRS medium (Oxoid CM561) and the plates incubated under anaerobic conditions at 37°C for about 3 days followed by counting the number of colony forming units (CFU) per g of sample. The initial counts of the two lactic acid bacteria were about 10^9 per g. The counts were reduced by 1-2 log units during the following two days and kept stable at about 10^7 per g for the following 8 days.

5. Quality assessment of the injected ryebread loaves

The injected loaves were assessed with regard to crumb structure, sensoric characteristics and crumb consistency. The crumb structure showed an even satisfactory pore structure. The bread had a rich smell of ryebread and a strong and rich taste of rye and linseed. The salt and leaven contributed to the overall desired taste but these ingredients did not dominate the taste.

The consistency was assessed to be soft and suitably "wet" and the bread did not stick to the knife when cut. This desired consistency was kept satisfactorily during the 10 days period. The bread also had a desirable "resistance" on chewing.

It was concluded that the injected loaves had sensoric and consistency characteristics which are typical for ryebread loaves of good quality.

EXAMPLE 2

Yeast-leavened retzel bakery product ("kringle") containing a mixture of Lactobacillus acidophilus, Bifidobacterium bifidum, Streptococcus faecium and Propionibacterium shermanii

The retzel product was made from a dough containing the following ingredients: wheat flour, vegetable and animal fat, skimmed milk, eggs, baker's yeast, sugar, salt and wheat starch. The following food additives were added: modified starch, emulsifying agents (E 322, E 471, E 475, E 472e), ascorbic acid, sodium carbonate, citric acid and flavouring agent.

The product contained a filling comprising: animal and vegetable fat, sugar, muscovado, raisins, apricot kernels,

soybean flour, acidified skimmed milk, starch, water, salt, egg white and whey powder.

Into 900 g of the baked and cooled product was injected 0.25 mL of separate suspensions containing *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Streptococcus faecium* and *Propionibacterium shermanii*, respectively

The suspension medium for the bacterial cultures was Tryptone Soy Broth (TSB) (Oxoid CM129) and each of the bacterial suspensions contained 1 g of bacterial cultures in 10 mL of the suspension medium. The *Lactobacillus acidophilus* culture used was the commercial frozen DVS (direct vat set) culture L. acidophilus La-5 (Chr. Hansen's Laboratorium Danmark A/S, Hørsholm, Denmark) which contains at least 6×10^{10} colony forming units (CFUs) per g. The *Bifidobacterium bifidum* culture was the frozen DVS culture Bifidobacterium Bb-12 (Chr. Hansen's Laboratorium Danmark A/S, Hørsholm, Denmark) containing at least 10^{11} CFUs per g. The *Streptococcus faecium* culture which was used for preparing a suspension was the freeze-dried DRI-VAC culture Streptococcus faecium CH-1 (Chr. Hansen's Laboratorium Danmark A/S, Hørsholm, Denmark) containing 5×10^{8} CFUs per g and the *Propionibacterium shermanii* culture used was the frozen DVS Propionic Acid Culture PS-1 (Chr. Hansen's Laboratorium Danmark A/S, Hørsholm, Denmark) containing 3×10^{9} per g.

Subsequent to the suspending of the cultures the suspensions were left to stand for resuscitation at 37°C for 1-2 hours and then injected into the retzel product at amounts of 0.25 mL of each suspension by means of a syringe and the injected product was kept at room temperature until determination of viable bacterial counts.

About 24 hours after the injection of the bacterial suspensions the contents of viable bacteria in a slice of about 1.5 cm thickness cut around the site of injection were determined according to the following methods:

About 10 g of the above slice of product was homogenized in saline in a Stomacher for about 4 minutes and appropriate tenfold serial dilution were made. The viable counts of *Lactobacillus acidophilus* and of *Bifidobacterium bifidum* were done essentially according to the method defined in Example 1. The viable count of *Streptococcus faecium* was done by plating onto blood agar (Blood agar base, Oxoid CM55 supplemented with 5% calf blood) followed by aerobic incubation for 24 hours at 37°C. The viable count of *Propionibacterium shermanii* cells was done by plating onto the above-defined blood agar and incubating anaerobically at 37°C for 48 hours.

The numbers of viable bacteria per g of the retzel product were as follows:

(i)	*Lactobacillus acidophilus*	4×10^7
(ii)	*Bifidobacterium bifidum*	4×10^7
(iii)	*Streptococcus faecium*	1×10^7
(iv)	*Propionibacterium shermanii*	3×10^7

EXAMPLE 3

A sponge cake ("sandkage") containing a mixture of Lactobacillus acidophilus, Bifidobacterium bifidum, Streptococcus faecium and Propionibacterium shermanii

The sponge cake having a total weight of 350 g contained the following ingredients: margarine, water, sugar, wheat flour, starch, egg powder and milk powder. The following food additives were added to the batter: leavening agents (diphosphate salt, sodium hydrogen carbonate), emulsifying agents (E 471, E 322, E 475) and aroma.

0.25 mL volumes of the suspensions as defined in Example 2 were injected as also defined in Example 2, and after about 24 hours the bacterial counts (CFUs per g) were determined

according to methods defined in Example 2 with the following results:

(i)	*Lactobacillus acidophilus*	2×10^3
(ii)	*Bifidobacterium bifidum*	1.2×10^5
(iii)	*Streptococcus faecium*	1×10^7
(iv)	*Propionibacterium shermanii*	2×10^3

EXAMPLE 4

White bread ("franskbrød") containing a mixture of Lactobacillus acidophilus, Bifidobacterium bifidum, Streptococcus faecium and Propionibacterium shermanii

The bread was prepared from a dough containing the following ingredients: Wheat flour, water, baker's yeast, animal and vegetable fat, caseinate, salt, sugar and glucose; and the following food additives: emulsifying agents (E 482, E 475, E 471, E 322) and ascorbic acid. After baking and cooling to a temperature below 70°C, 0.25 mL of the bacterial suspensions as defined in Example 2 were injected into a loaf of the bread having a weight of 300 g and the viable counts determined after about 24 hours according to the methods as also defined in Example 2. The results of these counts are shown below:

(i)	*Lactobacillus acidophilus*	4.3×10^5
(ii)	*Bifidobacterium bifidum*	4×10^7
(iii)	*Streptococcus faecium*	1×10^7
(iv)	*Propionibacterium shermanii*	3×10^7

EXAMPLE 5

Whole-meal white bread ("4-kornsbrød") containing a mixture of Lactobacillus acidophilus, Bifidobacterium bifidum, Streptococcus faecium and Propionibacterium shermanii

The bread was prepared from a dough consisting of wheat flour, water, coarsely comminuted rye grains, whole-meal wheat flour, baker's yeast, linseed, sesame seed, salt, sugar; and the following food additive ingredients: emulsifying agent (E 472e) and ascorbic acid. The dough was formed into loaves each having a weight of 520 g.

After baking and cooling to a temperature below 70°C, 0.25 mL of the bacterial suspensions as defined in Example 2 were injected into a loaf of the bread and the viable counts determined after about 24 hours according to the methods as also defined in Example 2. The results of these counts are shown below:

(i)	*Lactobacillus acidophilus*	2.4×10^6
(ii)	*Bifidobacterium bifidum*	5×10^7
(iii)	*Streptococcus faecium*	1×10^7
(iv)	*Propionibacterium shermanii*	3×10^7

EXAMPLE 6

Rolls ("rundstykker") containing a mixture of Lactobacillus acidophilus, Bifidobacterium bifidum, and Propionibacterium shermanii

The rolls were prepared from a dough consisting of wheat flour, water, baker's yeast, salt, sugar and eggs and a food additive ingredient mixture, Compound 80 (Fællesforeningen for Danmarks Brugsforeninger, Albertslund, Denmark). The dough was formed into rolls each weighing about 30 g and baked at 250 to 240°C for about 12 minutes.

After baking and cooling to a temperature below 70°C, 0.25 mL of the bacterial suspensions as defined in Example 2 were injected into each roll and the viable counts of the injected bacteria were determined after about 24 hours according to the methods as also defined in Example 2 except that a whole roll of about 30 g was used the sample and homogenized by means of the Stomacher. The results of these counts are shown below:

(i)	*Lactobacillus acidophilus*	4.5×10^5
(ii)	*Bifidobacterium bifidum*	7.4×10^7
(iii)	*Propionibacterium shermanii*	2×10^8

EXAMPLE 7

Whole-meal white bread ("grovbrød") containing a mixture of Lactobacillus acidophilus, Bifidobacterium bifidum, and Propionibacterium shermanii

This bread was prepared from a dough consisting of wheat flour, water, whole-meal wheat flour, wheat grains, whole-meal rye flour, baker's yeast, animal and vegetable fat, sugar, salt and starch; and the following food additives: emulsifying agents (E 471, E 472, E 322), phosphate (E 341) and ascorbic acid. The dough was formed into loaves of a weight of 450 g.

After baking and cooling the loaves to a temperature below 70°C, 0.25 mL of the bacterial suspensions as defined in Example 2 were injected into the bread and the viable counts of the injected bacteria were determined after about 24 hours and again after about 72 hours according to the methods as also defined in Example 2. The results of these counts are shown below:

		24 hours	72 hours
(i)	*Lactobacillus acidophilus*	4 x 10^6	3.8 x 10^6
(ii)	*Bifidobacterium bifidum*	6.5 x 10^7	3.2 x 10^5
(iii)	*Propionibacterium shermanii*	2.2 x 10^8	2 x 10^8

EXAMPLE 8

Dietary fiber-enriched rye bread containing viable yeast

This bread was prepared from a dough containing the following ingredients: leaven (water, whole-meal flour of rye, leaven culture), water, whole-meal flour of rye, whole rye grains, dried crumbs of rye bread, salt, dried leaven (wheat flour, wheat bran, water, leaven culture), wheat starch, yeast, malt extract, syrup. The dough was formed into loaves of 1600 g, baked and cooled to a temperature less than 70°C.

A suspension of baker's yeast (*Saccharomyces cerevisiae*) was prepared in the following manner: 1 g of the baker's yeast (Malteserkorsgær™, De Danske Spritfabrikker) was suspended in 10 mL of TSB and the suspension was kept for 60 minutes at 37°C and a sample was collected for determination of the viable yeast cell count (CFU per g) which was found to be about 1.8 x 10^9 CFU of yeast per mL. A loaf of the bread was injected essentially as described in Example 2 but using 1 mL of yeast suspension and kept at room temperature for about 24 hours.

After keeping, a slice of about 1.5 cm was cut and 10 g hereof around the site of injection was collected for the determination of the viable yeast count according to the following method: serial dilutions were prepared as described

in Example 2 and appropriate dilution plated onto Malt agar (Oxoid CM591) and the plates were incubated aerobically for 3 days at 25°C followed by counting of the yeast colonies.

The viable count of yeast in the slice of ryebread as examined was 1.4×10^8 per g.

CLAIMS

1. A method of preparing a bakery product containing viable microorganisms, comprising the following steps:

(1) preparing a baked product and cooling it to a temperature in the range of 0 to 70°C,

(2) preparing a suspension of microorganisms, containing 10^7 to 10^{12} viable organisms per mL,

(3) injecting into the baked product a volume of the suspension of microorganisms which is in the range of 2 to 20 mL per kg of product,

to obtain the baked product having a content of viable microorganisms which is in the range of 10^3 to 2×10^{10} per g.

2. A method according to claim 1 wherein the microorganisms are selected from a yeast species and a bacterial species.

3. A method according to claim 2 wherein the microorganisms are lactic acid bacteria selected from the group consisting of a *Lactobacillus* species, a *Bifidobacterium* species, a *Lactococcus* species, a *Streptococcus* species, a *Leuconostoc* species and a *Pediococcus* species.

4. A method according to claim 3 wherein the microorganisms are lactic acid bacteria which is a mixture of two or more lactic acid bacterial species.

5. A method according to claim 4 wherein the microorganisms are a mixture of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*.

6. A method according to claim 1 wherein the suspension of microorganisms is prepared by suspending 1 part of a concentrate of microorganisms containing 10^8 to 10^{13} cells per g, in 5 to 20 parts of an aqueous medium comprising at least one microbial nutrient and at least one salt.

7. A method according to claim 1 wherein the suspension of microorganisms contains 10^9 to 10^{11} organisms per mL.

8. A method according to claim 1 wherein the volume of the suspension of microorganisms injected into the baked product is in the range of 3 to 15 mL per kg of product.

9. A method according to claim 1 wherein the baked product is selected from a ryebread product, a yeast-leavened bakery product and a chemically leavened bakery product.

10. A method according to claim 1 wherein the suspension of microorganisms is injected into the baked product through a multiplicity of needles having a length which is in the range of 5 to 100 cm and a diameter being in the range of 0.5 to 3 mm, the needles being connected to a reservoir containing the suspension.

11. A method according to claim 1 wherein the suspension of microorganisms is injected longitudinally into the baked product.

12. A method according to claim 1 wherein the baked product being prepared is a rye bread prepared from a dough containing a leaven.

13. A method according to claim 1 wherein the suspension of microorganisms is injected by means of an apparatus comprising

(1) means for introducing the suspension into the baked product,

(2) means for containing the suspension to be injected,

(3) means for connecting the reservoir with said means for introducing the suspension,

(4) means for pneumatically transporting the suspension from the containing means and to the introducing means, and for pneumatically introducing the means for introducing the suspension into the baked product,

(5) means for adjusting the volume to be injected,

(6) means for maintaining the baked product to be injected in a position which allows the introduction of the suspension thereinto.

14. A method according to claim 13 wherein the means for introducing the suspension into the baked product is a multiplicity of needles.

15. A method according to claim 14 wherein the means for introducing the suspension into the baked product comprises a number of needles which is in the range of 2 to 20.

16. A method according to claim 14 wherein the needles have a length which is in the range of 5 to 100 cm.

17. A method according to claim 14 wherein the needles have a diameter which is in the range of 0.5 to 3 mm.

18. A method according to claim 14 wherein the needles have a diameter which is about 2 mm.

19. A method according to claim 13 wherein the means for containing the suspension is provided with agitating means.

20. A method according to claim 13 wherein the means for pneumatically transporting the suspension is activated during

introduction of the means for introducing the suspension into the baked product.

21. A method according to claim 13 wherein the means for adjusting the volume to be injected allows adjustment to a volume which is in the range of 3 to 15 mL.

22. A baked product having a content of viable microorganisms which is in the range of 10^3 to 2×10^{10} per g.

23. A baked product according to claim 22 wherein the microorganisms are selected from a yeast species and a bacterial species.

24. A baked product according to claim 23 wherein the microorganisms are lactic acid bacteria selected from the group consisting of a *Lactobacillus* species, a *Bifidobacterium* species, a *Lactococcus* species, a *Streptococcus* species, a *Leuconostoc* species and a *Pediococcus* species.

25. A baked product according to claim 24 wherein the microorganisms are lactic acid bacteria which is a mixture of two or more lactic acid bacterial species.

26. A baked product according to claim 25 wherein the microorganisms are a mixture of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*.

27. A baked product according to claim 22 wherein the suspension of microorganisms comprises 1 part of a concentrate microorganisms containing 10^8 to 10^{13} cells per g, in 5 to 20 parts of an aqueous medium comprising at least one microbial nutrient and at least one salt.

28. A baked product according to claim 22 wherein the injected suspension of microorganisms contains 10^9 to 10^{11} organisms per mL.

29. A baked product according to claim 22 wherein the volume of the suspension of microorganisms injected thereinto is in the range of 3 to 15 mL per kg of product.

30. A baked product according to claim 22 which is a product prepared by injecting the suspension of microorganisms into a baked product selected from a ryebread product made from a dough containing leaven, a yeast-leavened bakery product and a chemically leavened bakery product.

31. A baked product according to claim 30 which is a yeast-leavened baked product selected from a wheat flour-containing loaf product not containing rye flour, a roll and a bun.

32. A baked product according to claim 31 which is a chemically leavened product selected from a cake, a muffin and a scone.

INTERNATIONAL SEARCH REPORT

PCT/DK 93/00207

International Application N

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A21D15/00; A61K9/20; A23L1/03

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A21D ; A61K ; A23L

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
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P,Y	EP,A,0 512 857 (T. YABIKI) 11 November 1992 see page 3, line 28 - page 4, line 26; claims ---	1-5,10, 21-27
A	US,A,4 806 368 (M.S. REDDY) 21 February 1989 see abstract see column 1, line 22 - column 2, line 9; claims ---	1-6, 22-27
A	EP,A,0 154 549 (KABUSHIKI KAISYA ADVANCE KAIHATSU KENKYUJO) 11 September 1985 see page 23, line 12 - line 20; claims --- -/--	1-6, 22-27

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 OCTOBER 1993	14 -10- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	BEVAN S.R.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

DK 9300207
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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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